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Title: Casein phosphopeptide-amorphous calcium phosphate attenuates virulence and modulates microbial ecology of saliva-derived polymicrobial biofilms

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Running head: Ecological effects of CCP-ACP

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Declaration of interests

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Abstract

Background: Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) acts as a salivary biomimetic that provides bioavailable calcium and phosphate ions to augment fluoride-mediated remineralization of early caries lesions. However, there are indications that it may also have ecological effects on the oral microbiome.

Objective: This *in vitro* study investigated whether CPP-ACP could influence acidogenicity, microbial counts, and the relative abundance of specific caries-and health-associated bacterial species in polymicrobial biofilms.

Methods: Saliva-derived polymicrobial biofilms were grown for 96 h in a cariogenic environment and treated every 12 h with 2% CPP-ACP or vehicle control. Acidogenicity and colony forming units (CFUs) were estimated from the treated biofilms. Ecological effects of CPP-ACP was assessed based on the relative abundance of 14 specific caries- and health-associated bacterial species using a real-time quantitative PCR assay.

Results: CPP-ACP-treated biofilms showed significant reduction in acidogenicity (33% reduction, $P < 0.001$) and significantly fewer microbial CFUs ($P = 0.008$) compared to the control-treated biofilms. The CPP-ACP treated biofilms also exhibited significantly lower bacterial loads of *Scardovia wiggsiae* and *Provotella denticola,* and higher bacterial of *Streptococcus sanguinis*, *Streptococcus mitis*/*oralis*, and *Steptococcus salivarius*/*thermophilus*.

Conclusions: The results indicate that CPP-ACP has cariogenic virulence-attenuating attributes that can influence a beneficial microbial ecological change in the biofilm.

A symbiotic oral microbiome delivers important health benefits and plays a critical role in preventing dysbiosis-mediated oral diseases like dental caries [Marsh, 2018]. Dental caries is now widely recognised as a disease triggered by microbial dysbiosis in the plaque biofilm and maintaining ecological balance in dental plaque is critical for long-term control over the disease [Burne, 2018; Philip et al., 2018b]. While acids produced from bacterial glycolysis of dietary carbohydrates can favour the dominance of acidogenic/aciduric bacteria in the plaque biofilm, health-associated plaque microflora can re-establish the homeostatic balance by producing alkali metabolites to neutralise the acid [Nascimento, 2018]. This evolving view of ecological battles and polymicrobial synergies has important implications for developing effective therapeutics against dental caries [Bowen et al., 2018].

Fluoride is without doubt the gold standard among remineralizing agents used for caries prevention. However, recent clinical studies have shown that it has minimal effects on the microbiome ecology [Adams et al., 2017; Koopman et al., 2015; Reilly et al., 2016; Reilly et al., 2014]. This suggests the need for additional ecological measures to supplement the predominantly physicochemical cariostatic effects of fluoride, especially in high caries-risk individuals [Philip et al., 2018b]. Although biocides like chlorhexidine have been used in the past for caries control, their broad-spectrum of antimicrobial action tend to indiscriminately eliminate even health-associated commensals. A delicate balance has to be achieved to control oral microflora at levels compatible with health [Marsh et al., 2015]. Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), a naturally derived milk protein-based remineralizing technology, may hold greater promise in effecting a beneficial shift in the dental plaque microbial ecology. While there is a growing body of evidence that supports the use of CPP-ACP to augment remineralizing effects of fluoride [Philip, 2019], there have also been suggestions that it could also have beneficial ecological effects on dental plaque too [Philip and Walsh, 2018]. Several clinical trials have shown that regular use of CPP-ACP reduced mutans streptococci (MS) levels in saliva [Emamieh et al., 2015; Plonka et al., 2013; Pukallus et al., 2013; Subramanian and Naidu, 2009; Yetkiner et al., 2014]. This interference with cariogenic bacteria may beneficially modulate the virulence of dental plaque and enable health-associated commensals to thrive. However, previous studies have mostly used a single bacterial species (usually *Streptococcus mutans*) as the surrogate marker for dental plaque ecology. For ecological caries control, it is more relevant to know the influence of CPP-ACP on a range of health- and disease-associated bacteria. Hence, as a prelude to a clinical trial, this *in vitro* study was designed to investigate whether CPP-ACP could: 1) inhibit acidogenicity and microbial numbers of saliva-derived polymicrobial biofilms growing in a cariogenic environment; and 2) effect the relative abundance of specific caries- and health-associated bacterial species in these polymicrobial biofilms.

Materials and Methods

Test agent

A 15% aqueous solution of CPP-ACP was sourced from GC Corporation (Tokyo, Japan) and diluted to 2% for testing against the saliva-derived polymicrobial biofilms. The concentration was chosen to represent

what would be achieved in the oral cavity with the dilution by saliva of a topical crème or chewing gum containing 10% CPP-ACP. As the test solution of CPP-ACP contained a sodium benzoate preservative, a 0.1% (w/v) sodium benzoate solution was used for control comparisons.

Saliva collection

With institutional ethics approval (approval number 2017001492), parafilm-stimulated whole human saliva was collected from 16 caries-free healthy adult donors. The study participants did not brush their teeth the morning before saliva donation and abstained from food for 2 h prior to saliva donation. The donors and had no history of using antibiotics/mouthwashes in the previous 3 months. The collected saliva was pooled, diluted 2-fold in 60% sterile glycerol, dispensed into aliquots, and stored at -80°C.

Polymicrobial biofilm formation and treatments

The high-throughput Amsterdam Active Attachment (AAA) model [Exterkate et al., 2010] was used to grow the saliva-derived polymicrobial biofilms. The biofilms were grown for a total of 96 h on hydroxyapatite (HA) discs (9.5mm diameter x 2mm thick; Himed, New York, NY, USA). Briefly, the HA discs were fitted to the custom-made AAA model such that each HA disc fitted into one well of a 24-well flat-bottomed microtiter plate (Costar 3526, Sigma-Aldrich, New York, NY, USA). To partly replicate the salivary pellicle coating, the HA discs were initially conditioned with filter-sterilized clarified human saliva for 1 h at 37°C immediately prior to biofilm formation, as described previously [Philip et al., 2018a]. The saliva-coated HA discs (s-HA) were then moved to a 24-well plate containing 2 mL/well of 2% CPP-ACP or the vehicle control solution, and treated for 10 min. The pre-treated s-HA discs were shaken gently to remove excess treatment solutions, and transferred to a new 24-well plate containing the 2 mL/well of the biofilm inoculum and incubated anaerobically at 37°C in an 80 rpm orbital shaker for a total of 96 h. The inoculation medium was a 50-fold dilution of the pooled saliva in the mucin-rich McBain medium [McBain et al., 2005] supplemented with 1% (w/v) sucrose. The sucrose-containing growth medium was replenished every 8 h to provide a constant cariogenic challenge. Subsequent biofilm treatments took place every 12 h, until the end of the 96 h growth period. All treatment exposures were for 10 min, with the biofilm-laden HA discs dip-rinsed 5 times in phosphate buffered saline (PBS; Gibco, Thermo Fisher Scientific, Paisley, UK) prior to treatment to remove non-adherent cells. The biofilms were similarly rinsed post-treatment also to remove any residual treatment solutions.

Biofilm assays

At the end of the biofilm growth period, the effect of CPP-ACP on the acidogenicity was estimated using a lactic acid production assay, while effects on microbial viability were assessed by counting the colony forming units (CFUs) recovered from the treated biofilms. These biofilm assays were performed in triplicate, and repeated in three independent experiments. The ecological effects of CPP-ACP on the polymicrobial biofilms were assessed based on the relative abundance of certain specific caries- and health-associated

bacteria obtained from three independent duplicate biofilm experiments using a real-time quantitative PCR (qPCR) assay.

For estimating biofilm acidogenicity, the biofilm-bearing HA discs were placed in a 24-well plate containing 2 mL/well of buffered peptone water (Merck, Darmstadt, Germany) supplemented with 0.2% sucrose. The model was then incubated anaerobically for 3 h at 37°C to allow acid formation. The amount of lactic acid generated during this period was calculated by means of a standard curve colorimetric assay using the lactate dehydrogenase enzymatic method [Exterkate et al., 2010].

To assess microbial populations, the HA discs with the biofilms were gently detached from the AAA model lid and transferred to sterile tubes containing 1 mL PBS. The biofilms were dispersed by uniform vortexing at maximum speed for 1 min and a series of dilutions made. A 50 µL aliquot of the microbial suspensions was plated on tryptic soy agar blood plates using an automatic spiral plater (Autoplate; Advanced Instruments, Norwood, MA, USA) and incubated anaerobically at 37°C for 48 h. Microbial colonies were counted from the dilution that allowed visualization of distinct colonies and the CFU/mL values calculated after correcting for the dilution factor.

For the qPCR assay, the treated polymicrobial biofilms were dispersed as described previously for the CFU assay. Bacterial DNA was then extracted from the microbial suspension using the MO BIO Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following manufacturer's instructions. RNA was removed using RNAse A (Thermo Fischer Scientific, Scoresby, Australia) and the amount of isolated DNA was quantified spectrophotometrically. The bacterial load of 14 bacterial species were determined using a custom-made qPCR array (16 x 24 format; Qiagen, Hilden, Germany). The bacteria selected included 8 caries-associated bacterial species (*Actinomyces gerensceriae*, *Lactobacillus gasseri*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus parasanguinis*, *Scardovia wiggsiae*, *Veillonella parvula*, and *Provotella denticola*) and 6 health-associated commensal bacterial species (*Streptococcus sanguinis*, *Streptococcus mitis/oralis*, *Steptococcus salivarius/thermophilus*, *Corynebacterium durum*, *Rothia aeria/dentocariosa*, and *Neisseria flavescens*). The assay used the 16S rRNA gene of the relevant bacterium, with the proprietary probes designed using the GreenGene database for 16S sequences [DeSantis et al. 2006]. The DNA sample was mixed with a proprietary master mix and robotically dispensed into a 384-well plate (10µL, 7 ng DNA/well) containing freeze-dried primers and fluorogenic probes for each of the bacterial 16S rRNA genes tested. Arrays also contained a positive PCR control to test for inhibitors in the sample, and a non-template control to account for assay background. Reactions were performed with the 384-well plate QuantStudio™ 6 Flex Real-Time PCR sequence detection system (Thermo Fisher Scientific) with the following cycling conditions: enzymatic activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 2 min. Data was analysed using the sequence detection system software (QuantStudio v1.3; Thermo Fisher Scientific).

Statistical analysis

For the lactic acid assay and CFU counts, depending on the normality of data distribution, an independent sample t-test or the Mann-Witney *U*-test was performed to detect any statistical difference between the CPP-ACP and control-treated biofilms. For the qPCR assay, the fold change of each bacterial amplicon in CPP-ACP- versus control-treated biofilms was calculated using the comparative cycle threshold method (ΔΔCt). Briefly, for each sample, the Ct value of the individual bacterial species was normalized to the mean Ct value of all bacterial species ($\Delta Ct^{\text{bacteria species}} = Ct^{\text{bacteria species}} - Ct^{\text{mean of all bacterial species}}$). The ΔΔCt for CPP-ACP- versus control-treated biofilms was then calculated for each bacterial species as follows: $\Delta\Delta Ct = \Delta Ct^{\text{bacterial species (CPP-ACP)}} - \Delta Ct^{\text{bacterial species (control)}}$. Fold increase, or decrease, in abundance was calculated based on the formula $2^{-\Delta\Delta Ct}$. For each bacterial species, independent t-tests were performed to test for differences between the CPP-ACP- and control-treated biofilms using the ΔCt values (add ref) . *P*-values were considered significant only if they were less than the Simes critical *P*-value [Simes, 1986]. Statistical software SPSS version 25 (IBM, New York, NY, USA) was used to perform the analyses.

Commented [SL1]: Please add the following reference: Yuan, J. S., Reed, A., Chen, F. & Stewart, C. N., Jr. (2006) Statistical analysis of real-time PCR data. *BMC Bioinformatics* **7,** 85. doi:10.1186/1471-2105-7-85. This reference is required to support our analysis of the qpcr data.

Results

Biofilm microbial counts

Total CFUs recovered from the CPP-ACP-treated biofilms were significantly fewer ($P = 0.008$) than from the control-treated biofilms. The mean CFU counts were $7.9 \times 10^7 \pm 1.2 \times 10^7$ and $1.0 \times 10^8 \pm 1.1 \times 10^7$ for the CPP-ACP- and control-treated biofilms respectively (Fig. 1).

Biofilm acidogenicity

The polymicrobial biofilms treated with CPP-ACP demonstrated a significant reduction in acidogenicity compared to the control-treated biofilms (33% reduction, $P < 0.001$), with mean lactic acid concentrations of 3.6 ± 1.1 mM/L and 5.3 ± 1.2 mM/L respectively (Table 1).

Biofilm ecological effects

Compared to the controls, the CPP-ACP-treated biofilms had significantly lower abundance for two caries-associated bacterial species: *P. denticola* (fold change 0.005, $P < 0.001$) and *S. wiggsiae* (fold change 0.017, $P < 0.001$). All the other caries-associated bacteria (except for *L. gasseri*) also showed fold decreases, but did not reach statistical significance. Among the health-associated commensals, *S. sanguinis* (fold change 30.22, $P < 0.001$), *S. mitis/oralis* group (fold change 9.66, $P < 0.012$), and the *S. salivarius/thermophilus* group (fold change 89.35 $P < 0.001$), were detected significantly more abundantly in the CPP-ACP-treated biofilms than in the control-treated biofilms (Fig 2). *S. parasanguinis* was not detected in any of the biofilm samples and was not included in the comparison.

Discussion

Dental caries is a polymicrobial biofilm-mediated disease triggered by environmentally-induced dysbiosis in the resident oral microbiome [Marsh, 2015]. If the dysbiosis is not reversed it can lead to bacterial acid-mediated demineralization of the dental hard tissues and ultimately result in clinical cavitation. Comprehensive caries prevention protocols should therefore encompass not only agents that can affect the de-/remineralization balance, but also include measures that can maintain or restore the ecological equilibrium in the oral microbiome. The current study provides evidence of the ecological effects of CPP-ACP on complex polymicrobial biofilms, and extends the recent findings of CPP-ACP ecological effects on multispecies biofilm comprised of 6 bacterial species growing in a highly cariogenic environment [Dashper et al., 2018]. The present study also provides *in vitro* evidence that CPP-ACP can inhibit lactic acid production and microbial numbers in saliva-derived polymicrobial biofilms.

The AAA model used in this study has been shown to maintain reproducible, complex, and actively attached microbial biofilms in a habitat similar to dental plaque [Exterkate et al., 2010; ten Cate, 2015]. Pooled saliva from several individuals was used as the biofilm inoculum to improve the microbial diversity of the *in vitro* polymicrobial biofilms. A cariogenic environment was created by regular replenishment of the sucrose-supplemented growth medium. The 10 min treatment time was chosen as CPP-ACP crèmes are intended to remain in the oral cavity until salivary clearance. Selection of the bacteria for the qPCR assay was based on profile studies of bacterial species commonly associated with dental caries and health [Aas et al., 2008; Becker et al., 2002; Burne and Marquis, 2000; Gross et al., 2012; Kianoush et al., 2014; Richards et al., 2017; Takahashi, 2015; Tanner et al., 2016].

A small but significant decrease in the total CFUs recovered from the CPP-ACP-treated polymicrobial biofilms was seen in the current study. The lower microbial counts are likely to be due to the anti-adhesion effects of CPP-ACP, as the product is not known to have bactericidal or bacteriostatic effects. *In vitro* studies have demonstrated a rapid saturation of saliva-coated hydroxyapatite surfaces by casein complexes inhibiting the adherence of *S. mutans* and *S. sobrinus* [Erdem et al., 2011; Reynolds and Wong, 1983; Schüpbach et al., 1996; Vacca-Smith et al., 1994]. CPP-ACP was also shown to bind both to dental plaque (with a strong affinity for *S. mutans*) and to pellicle macromolecules adsorbed on the tooth surface [Reynolds et al., 2003; Rose, 2000b; Rose, 2000a]. The anti-adhesion effects of CPP-ACP may have a number of mechanisms. CPP-ACP is known to compete with free calcium for plaque calcium binding sites, reducing the degree of calcium bridging between the pellicle and adhering cells and between the bacterial cells themselves [Rose, 2000b]. CPP molecules can also mask the bacterial cell surface hydrophobic proteins and thereby impede the initial adhesion to pellicle-coated surfaces. The anti-adhesion effects of CPP-ACP could be the reason for the reduced levels of MS that have been observed in various clinical trials [Emamieh et al., 2015; Plonka et al., 2013; Pukallus et al., 2013; Subramanian and Naidu, 2009; Yetkiner et al., 2014]. Disruption of bacterial adhesion can impair the ability of cariogenic bacteria to recruit other key players during biofilm formation and prevent the establishment of a cariogenic microbiome [Simon-Soro and Mira, 2015].

The present study also demonstrated a significant, though relatively modest, reduction in biofilm acidogenicity in the CPP-ACP-treated biofilms compared to the control-treated biofilms. It is possible that treating the biofilms for a longer duration than the 96 h of this study could have resulted in greater reductions in biofilm lactic acid concentrations. In fact, several clinical studies have shown that regular use of CPP-ACP had pronounced buffering effects on salivary and plaque pH [Caruana et al., 2009; Heshmat et al., 2014a; Heshmat et al., 2014b; Marchisio et al., 2010; Ozdas et al., 2015; Peric et al., 2015]. The buffering mechanisms of CPP-ACP are likely to be due to its ability to act as a reservoir of peptides and phosphate ions which can offset any drop in biofilm pH [Philip and Walsh, 2018]. The catabolism of certain CPP amino acids (e.g. glutamine, asparagine) by bacterial peptidases would buffer against a pH fall through the production of ammonia [Reynolds and Riley, 1989]. The CPP phosphoseryl residues that are more resistant to hydrolysis readily accept protons and neutralize plaque acids [Reynolds, 1987]. Furthermore, the inorganic phosphate (PO_4^{3-}) and organic phosphate ($-O-PO_3^{2-}$) ions in the CPP-ACP nanocomplex can also contribute to elevating salivary/plaque pH [Dashper et al., 2018]. The buffering influence of CPP-ACP is thus an important virulence-attenuating attribute, creating a biofilm microenvironment that provides a selective ecological advantage to acid-sensitive commensal organisms.

The qPCR analysis provides evidence that CPP-ACP beneficially modulated the microbial ecology of the CPP-ACP-treated polymicrobial biofilms. In the present study, CPP-ACP was able to effect significantly lower relative abundance of *S. wiggsiae* and *P. denticola* compared to the control-treated biofilm. *S. wiggsiae* has been associated with severe early childhood caries and orthodontic white spot lesions [Tanner et al., 2011; Tanner et al., 2012], while *P. denticola* has been associated with initial enamel lesions [Chhour et al., 2005; Tanner et al., 2016; Torlakovic et al., 2012]. *S. mutans* also showed a fold decrease in the CPP-ACP-treated biofilm, but this did not reach the threshold for statistical significance. It has been suggested that when "heavyweight" aciduric bacteria such as *S. wiggsiae* begin to dominate, the relative numbers of *S. mutans* tend to decrease, as the biofilm pH is driven to below what can be tolerated by even *S. mutans* [Burne, 2018]. This could be the reason why significant differences in relative abundance of *S. mutans* were not seen between the CPP-ACP- and control-treated biofilms.

The three health-associated bacterial species (*S. sanguinis*, *S. mitis*, *S. salivarius*) that showed significant fold increases in the CPP-ACP-treated biofilms possess systems actively engaged in biofilm pH homeostasis. *S. sanguinis* and *S. mitis* are known to express arginine deaminase system (ADS) activity that can effectively neutralize acids produced from carbohydrate metabolism [Burne and Marquis, 2000; Nascimento, 2018]. *S. salivarius* is among the relatively small number of oral bacteria that encode urease enzymes that can play a role in elevating resting plaque pH [Burne et al., 2012].

Conclusion

A better understanding of oral microbial ecology, especially the importance of the balance between cariogenic and commensal microflora, has underlined the need to develop strategies to modulate the

microbial composition of dental plaque for caries prevention [Marsh et al., 2015]. Measures that help maintain a symbiotic oral microbiome would be particularly beneficial for long-term control over dental caries in high caries-risk patients. CPP-ACP may hold particular promise in this regard as not only can it boost the remineralizing effects of fluoride, but the results of this study indicate that it may have beneficial microbial ecological effects too. Future clinical trials are required to ascertain whether these *in vitro* effects can translate to a clinical environment.

Figure Legends

Fig 1. Box-plots of microbial populations recovered from the polymicrobial biofilms after treatment with the CPP-ACP/vehicle control (n = 9). * indicates significantly different CFU values ($P < 0.05$) for the CPP-ACP-treated biofilm compared to control-treated biofilms.

Fig. 2. Fold change of A) cariogenic and B) commensal bacterial species in CPP-ACP- vs. control-treated biofilms. A ratio below 1 indicates lower relative abundance; a ratio above 1 indicates higher relative abundance. * indicates statistically significant results after the Simes adjustment for multiple comparisons. *P*-values in brackets are from independent t-tests.

Conflict of interest

None

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